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In vitro regeneration and dry matter assessment of pyrethrin-rich *Tanacetum cinerariifolium* genotypes

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Abstract

Pyrethrins, a group of six closely related insecticidal esters derived from *Tanacetum cinerariifolium* (pyrethrum), are considered the safest natural pesticides for the environment. This study focused on the in vitro propagation of six naturally pyrethrin-producing genotypes of *T. cinerariifolium* through direct shoot regeneration using apical shoot explants. Highly pyrethrins reproducing genotypes were identified by regeneration technique using apical shoot. Significant variation was observed among genotypes in terms of plant height and number of leaves. The Chlorophyll A content of leaves at 60 days old varied significantly among pyrethrum genotypes. Data analysis showed that percent dry matter content was significantly difference between genotypes. The maximum percent dry matter content was observed in genotype LW 8-9 (9.03%) which was followed by genotype LW 9-10(8.75%) of *T. cinerariifolium*. These findings suggest that direct shoot regeneration is an effective method for selecting and propagating high pyrethrin-producing genotypes of *T. cinerariifolium* for potential use in sustainable pest management programs.

Keywords: *Insecticide, repellent, tanacetum cinerariifolium, root, shoot*

Introduction.

Natural pyrethrins producing plant, *Tanacetum cinerariifolium* belonging to the family Asteraceae commonly known as pyrethrum. The pyrethrins are produced all over the plant parts (Figure 1) but highly concentrated in the glandular trichomes of flower achenes (ssultana *et al.* 2015). The ability of natural pyrethrins to quick knock-down effect on a wide range of insect species and paralysis within a few minutes with minimal or no adverse ecological impact (Casida and Quistad 1995). It acts as contact poison and affects the central nervous system of insects by blocking their nervous functions (Grdisa *et al.* 2009). The term pyrethrins referred to the six insecticide active ingredients: pyrethrin I, cinerin I, jasmolin I, pyrethrin II, cinerin II and jasmolin II (Figure 2). Pyrethrins is about 0.1-0.2% (dry weight) in leaves and 1-2% (dry weight) in flowers (Baldwin *et al.* 1993). The inadequate supply of natural pyrethrins has led to comparatively high prices and an inability to meet the fast-growing demand. It is therefore essential to reduce cost by increasing the production of pyrethrins.

It is widely known that plants from a natural resource or plants propagated by conventional means generally produce small quantities of secondary metabolites which makes their price very high; hence the studies on plant tissues and plant micropropagation offer various advantages for obtaining massive amounts of plant material. The plant micropropagation is an efficient method for propagating disease-free and genetically uniform plants. The vegetative propagation of this plant is generally achieved by either splits or shoot cuttings. These propagation methods result in a low multiplication rate (Hitmi *et al.* 1998).

An additional problem is the high susceptibility of *T. cinerariaefolium* to attack by rootknot nematodes; the splitting of infected stock plants results in a contaminated clone (Wambugu and Rangan 1981). Besides offering increased rates of propagation and pathogen-free plants, in vitro clonal multiplication facilitates asexual multiplication of species that have desirable characteristics. Due to the higher cost compared to many other insecticides, the applications of natural pyrethrins are still restricted. There have been very few attempts to develop regeneration techniques for *T. cinerariifolium* (Zito *et al.* 1994). This study was carried out with the objective of enhancing pyrethrins production in different genotypes by direct shoot organogenesis.

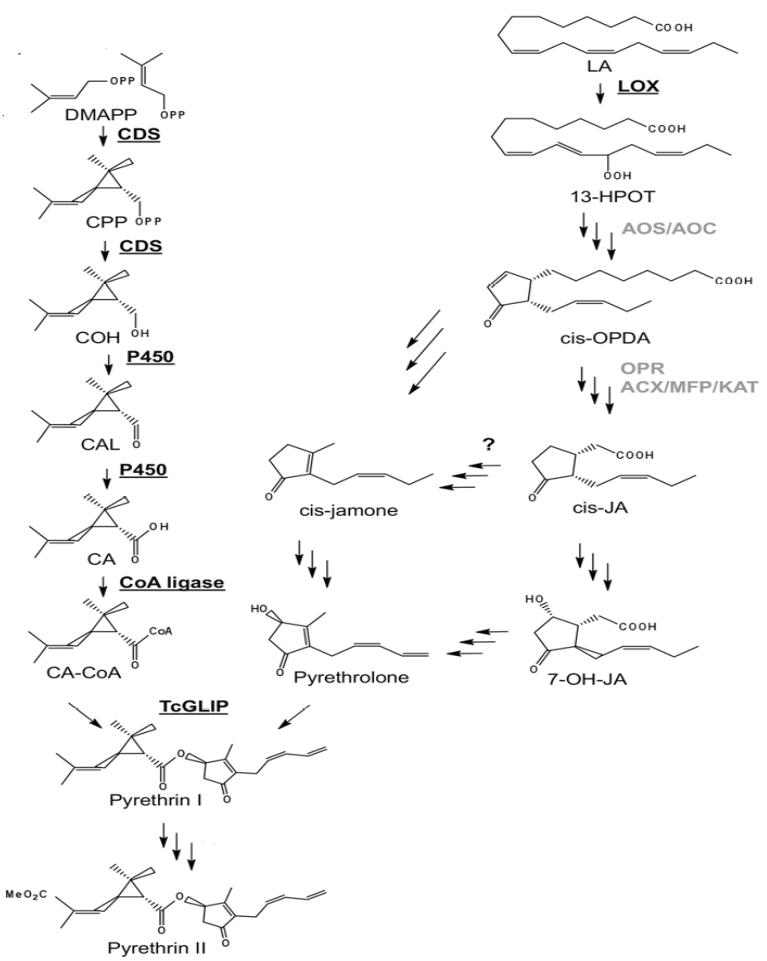


Figure 1. Biosynthesis pathway of pyrethrins (Sultana *et al.* 2015)

Materials and methods

Plant material and explant preparation: Seeds were collected from Honghe Senju Biology Co., Ltd. Luxi County, Yunnan Province, China and sown in plastic pot containing peat soil and transfer to culture chamber. The culture chamber environment is 12 hours light, 8 hours dark, and 2 hours semi-dark condition. One week later seedlings were transfer in a pot and placed in green house. Thirty plants were allowed to grow for two months. Two months old fresh leaves of six genotypes were excised and thoroughly washed with running tap water for about half an hour, treated with commercial detergent (2.0% Teepol) for 4-5 min and were rinsed well with distilled water. Fresh, young apical shoots of six genotypes LW1-1, LW2-3, LW4-5, LW7-8, LW8-9, and LW9-10 were selected for in vitro regeneration methods. The explants were thoroughly washed with running tap water for about half an hour, treated with commercial detergent (2.0% Teepol) for 4-5 min and were rinsed well with distilled water. Subsequently, these nodes were surface sterilized with 0.1% $HgCl_2$ for 3-5 min and thoroughly washed in sterile distilled water for 4-5 times.

Shoot induction: The explants were sub cultured on shoot induction medium supplemented with 2.0 mg/L 6-BA, 0.2 mg/L, IBA 1.0 mg/L at pH 5.8 under a photoperiod of 14 hours light (3000 lx) and 10 hours darkness (Sultana *et al.* 20155). The induced shoots were then sub-cultured on fresh shoot induction media at weekly intervals for 4 weeks. Shoots were then sub-cultured on shoot elongation medium for 4 weeks.





Figure 2. Pyrethrum flower and mosquito repellant from natural pyrethrins

Root induction and transfer to soil: Well-developed shoots were then transferred on rooting medium supplement with MS salts, vitamins, NAA (at suitable concentration), 3% (w/v) sucrose, and 0.8% (w/v) agar adjusted to pH 5.8. All cultures were maintained under the same environment conditions as described above.

Regenerated healthy plants from different genotypes were carefully removed from the culture bottles and agar was gently washed off the roots. Plants were then transferred to small pots containing a 1:1 (v/v) mixture of vermiculite and compost for acclimatization. They were maintained for further growth under 16 hours light/8 hours darkness at 22°c in a culture chamber before being moved to green house. This experiment was conducted following a complete randomized design (CRD) with three replications.

Phenotypic data analysis: For the direct regeneration of pyrethrum apical shoot, data were recorded for different parameters including average number of shoots per explant, average number of leaves per shoot and average number root per shoot. Six genotypes were grown in a greenhouse. Phenotypic data, including plant height and number of leaves per plant, were recorded.

Chlorophyll content: For analysis chlorophyll and carotinoid content, 200mg leaves were collected from two-month-old plant. Leaf samples were extracted and kept in the dark for 48 hours. The absorbance was measured at wave lengths of 665 nm and 649 nm using a spectrophotometer ((Lichtenthaler 1987).

Dry matter content: One month old plants were used for dry matter content measurements. Samples were immediately placed in polythene bag at 4°C for 24 hours in darkness and measured as soon as possible. Subsequently, samples were oven-dried in paper bags at 60°C for at least 72 hours, before their dry mass was measured.

Statistical analysis: All the experiments were repeated three times. Statistical significance was determined using Duncan's Multiple Range Test at $p<0.05$.

Results and discussion

It is widely known that production of pyrethrins from natural resource generally yields small quantities, which makes their price is very high. Hence, studies on plant tissues and plant micropropagation offer various advantages. To obtain massive amounts of plant materials within very short time many efforts have focused on enhancing the production of pyrethrins. Plant micropropagation provides an efficient method for propagating disease-free and genetically uniform plants.

Pyrethrum is naturally self-incompatible and highly heterozygous resulting in each seedling producing a different genotype. Thus, the development of an alternative system and the selection of suitable genotypes for the

rapid production of pyrethrum through tissue culture methods are crucial. The production of pyrethrum using tissue culture methods from leaf, flower and node were studied (Hitmi *et al.* 2000, Ikahu and Ngugi 1993). Shoot regeneration from calluses through organogenesis has been reported (Paul *et al.* 1988, Smith 1997). Artemisinin production in shoot cultures of such species as *A. pontica*, *A. judaica*, *A. vulgaris, A. annua,* and *A. scoparia* were investigated by a group of researchers (Gulati *et al.* 1996, Liu *et al.* 2004, Mannan *et al.* 2010, Mathur and Kumar 1996, Singh and Sarin 2010, Sujatha *et al.* 2007).

Selection of high pyrethrin-producing genotypes through regeneration techniques using apical shoots as explants was studied. After two weeks of explants cultured in all the genotype showed shoot bud initiation (Fig. 2). The maximum number of regenerated shoots were found from genotype LW7-8 (7.5) whereas minimum from genotype LW1-1 (5.5) (Table 1).

These results are supported by the observations of Bhattacharya *et al.* (1990), who also reported maximum (2.15) shoots per explant in chrysanthemum shoot tips when MS medium was supplemented with 0.1 mg/L IAA.

Maximum number of leaves per shoot and regenerated root were observed significantly different among genotypes. For root induction and its development in pyrethrum micro-shoots, half strength MS media supplemented with different concentrations of IBA and NAA were used. Karim *et al.* (2002) who reported that 0.2 mg/l IBA had produced maximum results regarding the rooting of chrysanthemum micro-shoots.

All phenotypic data, including plant height and number of leaves, showed significant differences among genotypes (Table 2). Sixty days old plants among pyrethrum genotypes varied significantly Chlorophyll A content of leaves (Table 2). The genotype LW8-9 showed maximum chlorophyll A content (1.01 mg/g fresh weight), while the lowest was obtained from genotype LW2-3 (0.54 mg/g fresh weight) (Table 2). Similarly, high chlorophyll

Table 1. Performance of six genotypes on average number of shoot/ explants, number of regenerated leaf/ shoot, number of regenerated root/ explants on culture medium at 28 days after culture.

Genotype	Average no of regenerated shoot/explant	No of regenerated leaf/shoot	No of regenerated root/shoot
LW1-1	5.5c	43.56b	8.67c
LW2-3	6.0b	45.34b	10.24b
LW4-5	5.6c	39.65c	10.12b
LW7-8	8.5a	46.32a	6.87d
LW8-9	6.6b	48.24a	9.45c
LW9-10	6.2b	45.69b	12.05a

Same letters on columns denote non-significant differences ($p \leq 0.05$)

Table 2. Comparisons of growth parameters among six genotypes (60 days old plant in green house).

Genotype	No. of leaves	Plant height	Chlorophyll A content (mg g^{-1} FW)	Chlorophyll B content (mg g^{-1} FW)
LW1-1	14.78	13.03b	0.78b	0.42c
LW2-3	14.63	15.30a	0.54c	0.38d
LW4-5	15.03	13.36b	0.98a	0.35b
LW7-8	15.56	13.56a	0.97a	0.52b
LW8-9	15.23	13.95a	1.01a	0.64a
LW9-10	14.87	13.63	0.79b	0.58d

Same letters on columns denote non-significant differences ($p \leq 0.05$)



Figure 3. Regeneration of plantlets from shoot tip of *Tanacetum cinerarrifolium*. A) Explants on shoot induction medium; B.C). Calli-derived shoot formation; D) Shoot elongation medium E) ; Plantlet on regeneration medium ; F) Plantlets transferred into rooting medium; H) Plant transferring to soil; G) Plants in pot; H) Well developed plants in green house.

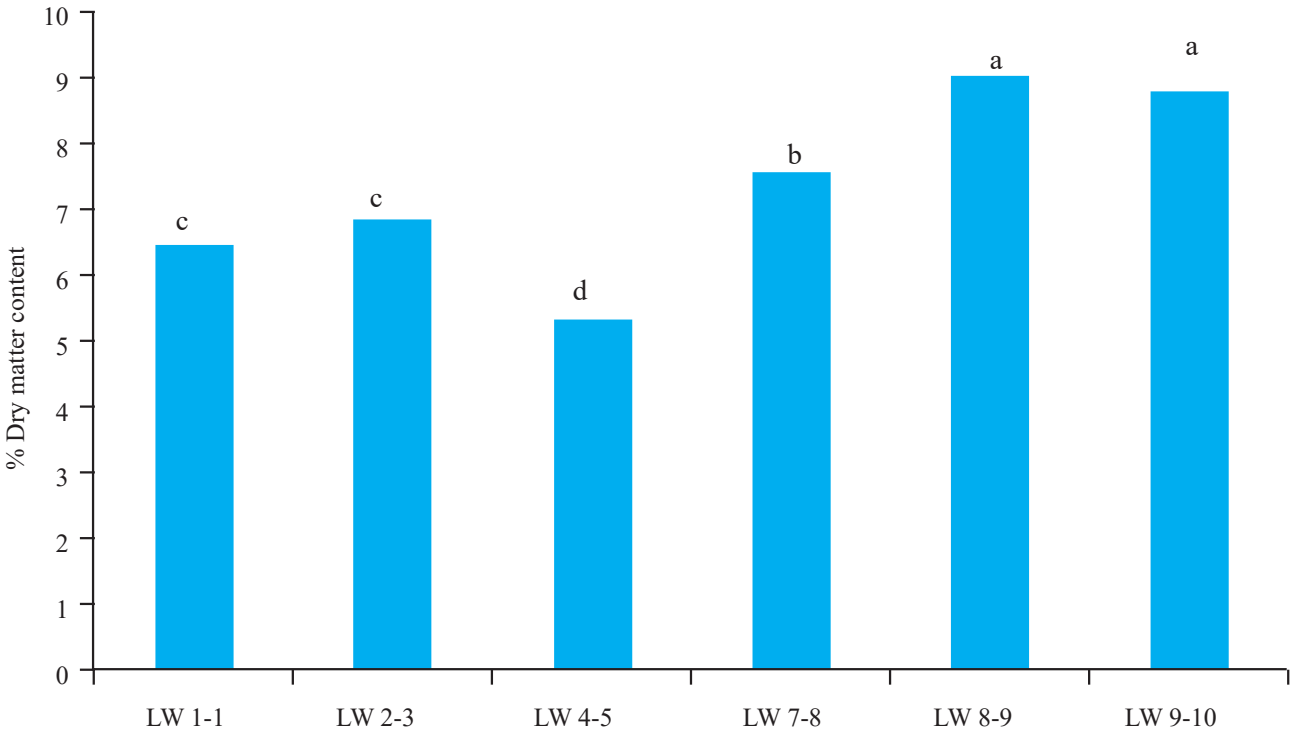


Figure 4. Percent dry matter content of *Tanacetum cinerariifolium* genotypes (LW1-1, LW2-3, LW4-5, LW7-8, LW8-9, and LW9-10)

B content was obtained in genotype LW 8-9 (0.64 mg/g fresh weight) and the lowest chlorophyll B were found in genotype LW 4-5 (0.35 mg/g fresh weight). Thomas and Lekharani, (2008) reported in orchid that chlorophyll content variation in might be due to the varietal characteristics. Increased chlorophyll content in leaves enhances photosynthetic activity, leading to higher carbohydrate production. In this study, percent dry matter content was also observed in six genotypes Significant variations were found in different genotype (Figure 3). Genotype LW8-9 showed the highest dry matter content (9.03%) followed by genotype LW9-10 (8.79 %). The lowest dry matter content was found in genotype LW4-5 which was 5.33%.

Conclusion

In the present study, shoots were regenerated from excised shoot tips. High yielding and disease-free plants produced apical shoots represent a better practice to increase pyrethrins production. In conclusion, based on the present investigation genotypes LW8-9 and LW9-10 appear promising for use as stock material for pyrethrins production.

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